ORIGINAL

TSCA NON-CONFIDENTIAL BUSINESS INFORMATION

DOCUMENT DESCRIPTION	DOCUMENT CONTROL NUMBER	DATE RECEIVED
FYI-13-01709	84130000012	4/16/13

COMMENTS:

DOES NOT CONTAIN CBI

THE LAW OFFICES OF JOHN B. HALLAGAN

1620 I Street, N.W. • Suite 925
Washington, D.C. 20006

353459

Tel: 202-331-2333 • Fax: 202-463-8998
Email: Hondobear@aol.com

TSCA Confidential Business Information Center (7407M)
EPA East - Room 6428 Attn: FYI
U.S. Environmental Protection Agency
1200 Pennsylvania Avenue, NW
Washington, DC 20460-0001

9 April 2013

Re: TSCA 8(e) notification for 3-acetyl-2,5-dimethylthiophene. CAS No. 2530-10-1.

Dear TSCA Section 8(e) Coordinator:

On behalf of my client, the Flavor and Extract Manufacturers Association of the United States (FEMA), we are reporting results from a study to test the ability of 3-acetyl-2,5-dimethylthiophene to induce gene mutation in the *lacZ* transgene in the liver and duodenum of treated transgenic (Muta™Mouse) mice. This substance's CAS number is 2530-10-1.

We are submitting information on this study pursuant to Section 8(e) of the Toxic Substances Control Act (TSCA). We are aware only of the use of 3-acetyl-2,5-dimethylthiophene as a flavoring substance and therefore as a food consistent with Section 201(f) of the Federal Food, Drug and Cosmetic Act and are therefore providing this information as an "FYI" submission under the requirements of TSCA 8(e) in case there are other non-food uses of this substance of which we are not aware. This information was received through a report from the contract laboratory that performed the study, Covance (attached). This study does not involve effects in humans. This notification does not contain confidential business information.

Study Summary

3-acetyl-2,5-dimethylthiophene was administered in corn oil by gavage for twenty-eight consecutive days to groups of male transgenic mice at dose levels of 120, 235, or 300/350 mg/kg/day. No clinical signs of toxicity were observed in any mice dosed with 3-acetyl-2,5-dimethylthiophene at the 120 or 235 mg/kg/day dose levels. At the highest dose level tested (300/350 mg/kg/day), clinical signs of toxicity were noted up to Day 5 of the study and included but were not limited to hunched posture and piloerection. No such signs were observed from Day 6 onwards.

CONTAINS NO CBI

A statistically significant, dose-related increase in mutant frequency (MF) was observed in the liver of treated mice. No significant increases in MF were observed in the duodenum of these mice. Small but statistically significant increase in micronuclei were observed in peripheral blood reticulocytes taken from the high dose animals on Day 4 and from the intermediate dose level mice on Day 31. These increase were not dose dependant and were not elevated compared to Day 1 observations.

It was concluded that 3-acetyl-2,5-dimethylthiophene induced mutation in the *lacZ* transgene in the liver of male Muta™Mice that had been dosed daily for twenty-eight days at up to 300 mg/kg/day. 3-acetyl-2,5-dimethylthiophene did not induce mutation in the duodenum of the same animals, nor did it induce an increase in micronucleated reticulocytes in the peripheral blood.

Please contact me if you have any questions or require additional information. My direct telephone number is 202.331.2333.

Sincerely,

John B. Hallagan

Attachment – Study report

Final Report

Study Title	Induction of *lacZ*⁻ mutations in the liver and duodenum of treated Muta™Mice
Test Article	3-acetyl-2,5-dimethylthiophene
Author	C Beevers PhD
Sponsor	International Organisation of the Flavor Industry Avenue des Arts, 6 B-1210 Brussels Belgium
Study Monitor	Dr S Taylor
Test Facility	Covance Laboratories Ltd Otley Road, Harrogate North Yorkshire HG3 1PY, ENGLAND
Covance Client Identifier	1000243
Covance Study Number	8264099
Report Issued	March 2013
Page Number	1 of 63

COVANCE
THE DEVELOPMENT SERVICES COMPANY

STUDY DIRECTOR AUTHENTICATION AND GLP COMPLIANCE STATEMENT

3-acetyl-2,5-dimethylthiophene: Induction of *lacZ* mutations in the liver and duodenum of treated Muta™Mice

I, the undersigned, hereby declare that the work was performed under my supervision and that the findings provide a true and accurate record of the results obtained.

The study was performed in accordance with the agreed protocol and with Covance Laboratories Limited Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved.

The study was conducted in compliance with the United Kingdom Good Laboratory Practice Regulations 1999, Statutory Instrument No. 3106 as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004 and the OECD Principles on Good Laboratory Practice (revised 1997, issued January 1998) ENV/MC/CHEM (98) 17.

C Beevers PhD
Study Director

26 March 2013
Date

QUALITY ASSURANCE STATEMENT

3-acetyl-2,5-dimethylthiophene: Induction of *lacZ* mutations in the liver and duodenum of treated Muta™Mice

This study has been reviewed by the GLP Quality Assurance Unit of Covance and the report accurately reflects the raw data. The following inspections were conducted and findings reported to the Study Director (SD) and associated management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a "process" inspection programme. This can be in addition to phases scheduled on an individual study basis. Selected process inspections conducted and considered applicable to this study are included below.

In addition to the inspection programmes detailed below, a facility inspection programme is also operated. Details of this programme, which covers all areas of the facility annually (at a minimum), are set out in standard operating procedures.

Inspection Dates		Phase	Date Reported to SD and SD Management
From	To		
11 Jun 2012	11 Jun 2012	Protocol Review	11 Jun 2012
14 Jun 2012	14 Jun 2012	Protocol Amendment Review	14 Jun 2012
06 Aug 2012	06 Aug 2012	Protocol Amendment Review	06 Aug 2012
08 Aug 2012	08 Aug 2012	Dose Administration	08 Aug 2012
10 Aug 2012	10 Aug 2012	Protocol Amendment Review	10 Aug 2012
08 Oct 2012	08 Oct 2012	Sample Preparation	08 Oct 2012
14 Dec 2012	14 Dec 2012	Protocol Amendment Review	14 Dec 2012
20 Dec 2012	04 Jan 2013	Draft Report and Data Review	04 Jan 2013
20 Mar 2013	20 Mar 2013	Sign off Inspection Record	20 Mar 2013
25 Mar 2013	25 Mar 2013	Final Report Review	25 Mar 2013

Inspection Dates		Process Phase	Date Reported to SD and SD Management
From	To		
20 Jun 2012	20 Jun 2012	Historical Control Ranges	20 Jun 2012
04 Jul 2012	04 Jul 2012	Data Collation and Transfer	04 Jul 2012
17 Jul 2012	17 Jul 2012	Necropsy	17 Jul 2012
24 Jul 2012	24 Jul 2012	Stock Solution Preparation	24 Jul 2012
26 Jul 2012	26 Jul 2012	Stock Solution Preparation	26 Jul 2012
26 Jul 2012	26 Jul 2012	Dose Preparation	26 Jul 2012
01 Aug 2012	01 Aug 2012	Cell line checks	24 Aug 2012

M Somerset
Quality Assurance Unit

26 March 2013
Date

REVIEWING SCIENTIST'S STATEMENT

3-acetyl-2,5-dimethylthiophene: Induction of *lacZ* mutations in the liver and duodenum of treated Muta™Mice

I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.

L Lillford

L Lillford BSc MSc
Scientist

18 March 2013
Date

RESPONSIBLE PERSONNEL

3-acetyl-2,5-dimethylthiophene: Induction of *lacZ* mutations in the liver and duodenum of treated Muta™Mice

The following personnel were responsible for key elements of the study:

Study Director	C Beevers
Study Supervisor	G Pearce
Animal House Supervisor	A Wronska
Statistics	J Saul
Study Monitor [1]	S Taylor

[1] On behalf of the International Organisation of the Flavor Industry; located at Verto Solutions, LLC, Washington DC, USA.

ARCHIVE STATEMENT

3-acetyl-2,5-dimethylthiophene: Induction of *lacZ* mutations in the liver and duodenum of treated Muta™Mice

All primary data, or authenticated copies thereof, will be retained for 5 years in the Covance Laboratories Limited archives after issue of the Final Report. At this time, the Study Monitor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. The Study Monitor will be notified of the financial implications of each of these options at that time. One copy of the protocol and final report will be held in the Covance Laboratories Limited archives as per Covance company policy.

Frozen plasma samples will be retained at the test facility in appropriate freezer locations for at least 5 years from the date of report finalisation. Any remaining DNA samples will be retained for 1 year, whilst remaining frozen tissue samples will be retained for 5 years, in the Department of Genetic and Molecular Toxicology at Covance Laboratories Limited after issue of the Final Report. After 1 year DNA samples will be deemed unsuitable for further use and will discarded on approval of the Study Director. After 5 years, the Study Monitor will be contacted to determine whether the retained plasma/tissue samples should be transferred to an alternative storage area, retained or destroyed on their behalf.

ABBREVIATIONS

°C	Degrees Celsius
ANOVA	Analysis of variance
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECHA	European Chemicals Agency
ENU	Ethylnitrosurea
EU	European Union
g	Gram
g	Gravity
GLP	Good laboratory practice
IWGT	International Working group on Genotoxicity Testing
kg	Kilogram
LB	Luria broth
M	Molar
µg	Microgram
µL	Microlitre
mg	Milligram
mL	Millilitre
MF	Mutant frequency
MN	Micronucleated
MTD	Maximum tolerated dose
NaCl	Sodium chloride
NCE	Normochromatic or mature erythrocyte
OECD	Organization for Economic Cooperation and Development
P-gal	Phenyl-galactose
PBS	Phosphate buffered saline
pfu	Plaque forming unit
QA	Quality Assurance
RET	Reticulocyte
SD	Study Director
SDS	Sodium dodecyl sulfate
SOP	Standard operating procedure
TRAID	Transgenic Rodent Assays Information Database

CONTENTS

SUMMARY

3-acetyl-2,5-dimethylthiophene was tested for its ability to induce gene mutation in the *lacZ* transgene in the liver and duodenum of treated male transgenic mice.

Strain / Species:	Muta™Mice (CD_2-lacZ80/HazfBR).
Vehicle:	Corn oil.
Administration route:	Oral gavage.
Dosing regime:	Range-Finder Experiment: Test article administered daily for 7 consecutive days. Main Experiment: Test article and vehicle control administered daily for 28 consecutive days.
Gender:	Males only, as no gender differences observed in the Range-Finder Experiment.
Dose levels:	120, 235, 300/350 mg/kg/day. High dose group animals initially dosed at 350 mg/kg/day but due to adverse clinical signs the dose was reduced to 300 mg/kg/day from Day 3 onwards.
Maximum dose:	Maximum tolerated dose based on Range-Finder data.
Positive control:	No concurrent positive control animals treated. Tissue matched positive control DNA was included on all packaging occasions.
Animals per group:	Range-Finder Experiment: Three Main Experiment: Six Satellite animals: Three.
Dose volume:	10 mL/kg.
Clinical signs of toxicity:	No clinical signs of toxicity were observed in any animals dosed with vehicle or 3-acetyl-2,5-dimethylthiophene at 120 or 235 mg/kg/day. At the highest dose clinical signs of toxicity, which included but were not limited to hunched posture and piloerection, were observed in all animals up to Day 5. With one exception, no clinical signs were

	observed from Day 6 onwards. Severe clinical signs were observed in one high dose animal on Day 7; this animals was subsequently killed *in extremis*.
Tissues sampled:	Peripheral blood samples for micronucleus assessments on Day -1, Day 4 and Day 31 (the latter at necropsy).
	The liver and duodenum were taken from all animals on Day 31 (i.e. 3 days after the final test article administration).
Assay validity:	The concurrent vehicle control data were comparable with the laboratory's historical control data.
	Concurrent positive controls (mutation data) or biological controls (micronucleus data) confirmed correct functioning of the test system.
	The assay was therefore accepted as valid.

A statistically significant, dose-related increase in mutant frequency (MF) was observed in the liver (analysis performed using ANOVA at the 5% level) of treated mice. No significant increases in MF were observed in the duodenum of these animals. Small but statistically significant increases in micronuclei (analysis performed using Wilcoxon Rank Sum test at the 5% level) were observed in peripheral blood reticulocytes taken from the high dose animals on Day 4 or the intermediate dose animals on Day 31. These increases were not dose-dependent and were not elevated compared to the Day-1.

It was concluded that 3-acetyl-2,5-dimethylthiophene induced mutation in the *lacZ* transgene in the liver of male Muta™Mice that had been dosed daily for 28 days at up to 300 mg/kg/day (an estimate of the maximum tolerated dose). 3-acetyl-2,5-dimethylthiophene did not induce mutation in the duodenum of the same animals, nor did it induce an increase in micronucleated reticulocytes in the peripheral blood.

OBJECTIVE

The objective of this study was to evaluate the potential of 3-acetyl-2,5-dimethylthiophene to induce gene mutations in the *lacZ* transgene within liver and duodenum from treated Muta™Mice.

INTRODUCTION

The Muta™Mouse (*lacZ/galE*) assay was developed in 1989 (Gossen *et al.*, 1989) and further refined by the development of a positive selection system developed by Ingeny BV in The Netherlands (Gossen & Vijg, 1993) and described by Dean & Myhr (1994).

The Muta™Mouse (*lacZ/galE*) assay is one of the few *in vivo* genotoxicity assays that is capable of detecting the induction of point mutations and small deletions (as opposed to gross chromosomal damage/loss) and has been widely demonstrated to detect mutation in a range of tissues using known mutagens/carcinogens (Lambert *et al.*, 2005). The ability to detect such changes in a variety of tissues, means this assay is useful for evaluating the genotoxic potential of chemicals that might interact at the site of contact (Dean *et al.*, 1999) and therefore has distinct advantages over conventional *in vivo* mutagenicity tests in which exposure of an appropriate target tissue or site of contact to the test article cannot always be guaranteed.

The genome of the Muta™Mouse strain contains 40 copies of a transgenic lambda gt10 vector, each of which contains a bacterial *lacZ* gene, and is therefore present in every DNA-bearing cell of the animal. Treatments are performed *in vivo*, with sufficient treatment and expression time to permit any mutations to be expressed. Tissues are taken at necropsy, cellular DNA extracted and subsequently packaged into lambda bacteriophage. The packaged bacteriophage are then used to transfect a culture of *E. coli* C *lac*$^-$ *galE*$^-$ Kan^r (*galE*$^-$ Amp^r). Only bacteriophage units containing a *lacZ* gene from the transgenic vector in the mouse DNA are viable and capable of transfecting a bacterial cell. Successful transfection enables replication of the bacteriophage and transfection of neighbouring bacterial cells. This results in the formation of a visible plaque, that is an area of bacterial cell lysis in a lawn of uninfected viable bacterial cells. Furthermore, due to the *galE*$^-$ status of the host bacterial strain cells, only cells infected with a non-functional (i.e. mutated) *lacZ* gene can form plaques on the positive selection plates.

Mutant frequency (MF) is calculated using the total number of viable bacteriophage (i.e. phage that have been successfully packaged with the *lacZ* transgene and visible as plaques on titre plates), compared with the number of bacteriophage which contain mutated *lacZ* transgenes (i.e. plaques on positive selection plates).

This study was performed according to the protocol and four amendments with the exception of the minor deviations detailed in Appendix 4, which did not prejudice the validity of the study in anyway.

The study was initiated on 11 June 2012. Experimental work started on 18 June 2012 and was completed on 24 November 2012. The study completion date is considered to be the date the Study Director signs the final report.

EXPERIMENTAL DESIGN

Regulatory test guidelines

The test methodology used in this study was in accordance with the OECD Guideline 488 (OECD, 2011), the Report of the Transgenic Working group (TWG) at the 2002 International Workshop on Genotoxicity Testing (IWGT: Thybaud *et al.*, 2003) and current literature (as reviewed by Lambert *et al.*, 2005).

Justification for selection of the test system

Transgenic rodent mutation assays, such as Muta™Mouse are recommended by various regulatory authorities as an appropriate test to determine the genotoxic potential of a compound *in vivo* (COM, 2000; ICH S2(R1), 2011).

Muta™Mouse was selected as the test system as it is the most commonly reported transgenic system in use and forms the majority of entries in the Transgenic Rodent Assays Information Database (TRAID; Lambert *et al.*, 2005). There is also a large volume of background data in this transgenic strain in this laboratory.

Liver was selected for analysis as this tissue was expected to be highly exposed following oral administration and would also be exposed to any metabolites. Duodenum was selected as this is a rapidly dividing tissue and was one of the first sites of contact following oral administration.

Test article and vehicle control administration

All treatments were given via oral gavage in order to maximise exposure of the target organ to the test article and as this is the most likely route of human exposure. Animals were not fasted prior to dose administration.

In the Range-Finder Experiment, animals were dosed on each of 7 consecutive days.

In the Mutation Experiment, animals were dosed on each of 28 consecutive days (Day 1-28) and sacrificed on Day 31 i.e. 3 days after the final administration (OECD, 2011; Lambert *et al.*, 2005). The exceptions to this were two high dose animals (animals 20 and 110, which were not dosed for two days (animal 20: not dosed on Days 3 and 4; animal 110 not dosed on Days 2 and 3) due to adverse clinical signs.

Animals were administered the test article or vehicle control at a dose volume of 10 mL/kg. Individual dose volumes were based on individual body weight.

Justification for dose selection

The LD_{50} after intraperitoneal injection in mice was reported as 260 mg/kg/day. Based on this information an initial dose of 200 mg/kg/day was administered in a Range-Finder Experiment. Subsequent higher doses, up to 700 mg/kg/day were tested until an estimate of the maximum tolerated dose (MTD) was determined (OECD, 2011; ICH-S2R1, 2011).

From the results of the Range-Finder Experiment dose levels of 120, 235 and 350 mg/kg/day 3-acetyl-2,5-dimethylthiophene (equivalent to approximately 33% MTD, 66% MTD and MTD respectively) were selected for testing in the Main Experiment.

The maximum dose level selected was considered to be an estimate of the maximum tolerated dose (MTD). Covance Laboratories Ltd defines the MTD as the highest tested non-lethal dose level that induces clear evidence of toxicity, such that a significantly higher dose (e.g. a fold increase of 1.4) would be expected to cause lethality, morbidity or severe toxicity. A 1.4-fold dose interval is considered sufficiently small to conclude that a completely non-toxic dose could be the MTD if a dose 1.4-fold greater induced lethality, morbidity or severe toxicity.

Both male and female mice were used in the Range-Finder Experiment. In the absence of substantial inter-sex differences in toxicity (a difference in MTD of 2-fold or greater), or likely sex-specific human exposure, the Mutation Experiment may be conducted in a single sex. As there were no substantial inter-sex differences in toxicity, male animals only were tested in the Mutation Experiment (OECD, 2011).

Dose levels

The following dose levels were tested in this study:

Table 1: Dose Levels - Range-Finder Experiment

Group No.	Group Description	Dose level (mg/kg/day)	No of animals tested
1	3-acetyl-2,5-dimethylthiophene	200	3M 3F
2	3-acetyl-2,5-dimethylthiophene	700	3M 3F
3	3-acetyl-2,5-dimethylthiophene	350	3M 3F
4	3-acetyl-2,5-dimethylthiophene	500	3M 3F

M Male
F Female

Table 2: Dose Levels - Main Experiment

Group No.	Group Description	Dose level (mg/kg/day)	Animal ID	Days of administration
1	Vehicle [a]	0	1-6M	1-28
2	3-acetyl-2,5-dimethylthiophene	120	7-12M	1-28
3	3-acetyl-2,5-dimethylthiophene	235	13-18M	1-28
4	3-acetyl-2,5-dimethylthiophene	350	19-24M	1-2
		300		3-28
			Toxicokinetic animals	
1	Vehicle [a]	0	101-103M	1-28
4	3-acetyl-2,5-dimethylthiophene	350 (Day 1&2) 300 (Day 3-28)	104-112M	1-28

M Male
[a] corn oil

Adverse clinical signs were observed in Group 4 animals. As a consequence animal 20 was not dosed on Day 3 or Day 4 and animal 110 was not dosed on Day 2 or Day 3. Although the remaining animals in Group 4 displayed some clinical signs, these were not too severe and did not prevent further dose administration. However, given the observations seen, it was considered impractical to continue dosing Group 4 animals at 350 mg/kg/day. Consequently, the maximum dose level was reduced to 300 mg/kg/day from Day 3 onwards.

Tissues for mutation analysis

The following tissues were retained for immediate analysis of mutant frequency:

Liver
Duodenum.

In addition, samples of spermatozoa from the vas deferens and developing germ cells (from seminiferous tubules) were taken but not analysed. These tissues were retained for possible future analysis.

Peripheral blood was taken on Day -1, Day 4 and Day 31 (at necropsy) for micronucleus analysis.

Proof of exposure

Groups of male satellite animals were dosed with vehicle or 3-acetyl-2,5-dimethylthiophene (high dose). Plasma was isolated from these animals and stored frozen (<-50°C). No bioanalysis of the plasma was conducted under this study.

TEST AND CONTROL ARTICLES

Test article

3-acetyl-2,5-dimethylthiophene, also known as sample ID 00198880, batch number 0004489289 was a yellow liquid. It was received on 21 May 2012 and stored at 15-25°C protected from light. Purity was stated as 99% and expiry date was given as February 2013. The certificate of analysis provided by the Sponsor is given in Appendix 3. The test article information and certificate of analysis provided by the Sponsor are considered an adequate description of the characterisation, purity and stability of the test article. Determinations of stability and characteristics of the test article were the responsibility of the Sponsor.

Controls

The negative (vehicle) control group consisted of animals dosed with corn oil using the same dosing regime and dose volume used for the test article treated animals.

Untreated controls were not required as this vehicle has been tested previously in this laboratory.

Concurrent positive control animals were not included in this study. Tissue matched positive control DNA was included in all packaging reactions in order to confirm correct assay functioning. The positive control DNA originated from animals dosed with ethylnitrosurea under Covance Laboratories Ltd. GLP study 8259749. For the liver, positive control DNA extracted under study 8259749 was used. For the duodenum, positive control DNA was extracted under this study from tissue taken under study 8259749.

TEST ARTICLE FORMULATION

Preparation

Dosing preparations were freshly prepared prior to each dosing occasion by formulating 3-acetyl-2,5-dimethylthiophene in corn oil as follows.

The test article was weighed and added to the correct amount of vehicle and stirred to mix.

The following concentrations of 3-acetyl-2,5-dimethylthiophene were used during this study:

Table 3: 3-acetyl-2,5-dimethylthiophene Concentrations Tested

Experiment	Concentration of dosing preparation (mg/mL)	Dose administered (mg/kg/day)
Range-Finder	20.00	200
	35.00	350
	50.00	500
	70.00	700
Mutation Experiment	12.00	120
	23.50	235
	30.00	300
	35.00[a]	350[a]

[a] Day 1 and 2 of dosing only

Stability

No stability information was available at the time of animal dosing. Consequently, all formulations were stored at 15-25°C, protected from light, and used within 2 hours of preparation.

Homogeneity

To ensure homogeneity, dose formulations were stirred continuously (on a magnetic stirrer) immediately before and throughout dosing.

TEST SYSTEM

Species, strain and supplier

60 (48 male, 12 female) out-bred young adult Muta™Mouse strain, designated CD_2-lacZ80/HazfBR were purchased from Harlan, UK. This random bred mouse strain was designed specifically for the detection of mutations in the *lacZ* gene in a lambda gt10 transgene.

Specification

24 animals were dosed during the Range-Finder Experiment. They were in the region of eight to ten weeks old on the first day of dosing and in keeping with the weight range used in the Main Experiment.

Table 4: Animal Specification

	Mutation Experiment
Number of animals used in study	36M[a]
Weight range on first day of assay (g)	22-28
Approximate age on first day of dosing (weeks)	8-11[b]

M Male.
[a] Includes 12 satellite animals for bioanalysis.
[b] See minor deviations from protocol, Appendix 4.

Environment

The animals were routinely kept in the following environment except for short periods of time where experimental procedures dictated otherwise. The animals were housed in a room air-conditioned to provide 15-20 air changes/hour. The temperature and relative humidity ranges were 20 to 24°C and 45 to 65%, respectively. Fluorescent lighting was controlled automatically to give a cycle of 12 hours light (0600 to 1800) and 12 hours dark. The study room was used to house animals allocated to other studies.

The animals were housed in groups of up to three of the same sex in cages that conformed with the 'Code of practice for the housing and care of animals used in scientific procedures' (Home Office, London, 1989).

Environmental enrichment

In order to enrich both the environment and the welfare of the animals, they were provided with wooden Aspen chew blocks.

Diet, water and bedding

Throughout the study the animals had access *ad libitum* to SQC Rat and Mouse Maintenance Diet No 1, Expanded (Special Diets Services Ltd. Witham). Each batch of diet was analysed for specific constituents and contaminants.

Mains water was provided *ad libitum* via water bottles. The water is periodically analysed for specific contaminants.

Bedding was provided on a weekly basis to each cage by use of clean European softwood bedding (Datesand Ltd, Manchester). The bedding was analysed for specific contaminants.

No contaminants were expected to be present in diet, water or bedding at levels that might interfere with achieving the objective of the study. Results of analyses performed on diet, water, bedding and environmental enrichment are held centrally at Covance Laboratories Ltd.

Peripheral blood flow cytometric analysis kits

MicroFlow®PLUS in vivo micronucleus flow cytometry kits (mouse blood) were purchased from Litron Laboratories, Rochester, USA. These kits contained the following components:

- Blood fixative
- Anticoagulant
- Buffer solution
- RNase
- Mouse anti-CD71 antibody
- DNA stain (propidium iodide; PI)
- Platelet antibody
- 3x Biological standards for flow cytometer calibration.

Host bacteria for transfection

The proprietary strain of *E. coli* C *lac*$^-$ *galE*$^-$ Kan^r (*galE*$^-$ Amp^r) was developed and supplied by J Gossen and J Vijg, Ingeny BV, Leiden, The Netherlands.

DNA extraction kits

Recoverease™ DNA isolation and purification kits were obtained from Agilent Technologies UK Ltd, Stockport, UK (see minor deviations from protocol, Appendix 4).

Bacteriophage packaging extract and transfection reactions

Transpack kits, providing necessary buffers and reagents for DNA packaging into bacteriophage "heads" and subsequent transfection into the host bacteria, were obtained from Agilent Technologies UK Ltd, Stockport, UK (see minor deviations from protocol, Appendix 4).

METHODS

Pre-experimental procedures

Acclimatisation and health procedures

All animals were given a clinical inspection for ill health on arrival. They were acclimatised for at least 5 days and a veterinary inspection was performed before the start of dosing to ensure their suitability for study.

Allocation to treatment group

On arrival animals of the same sex were randomly allocated to cages. Range-Finder and satellite animals were allocated to groups of up to three and Main Experiment animals were randomised to groups of six.

Checks were made prior to dosing to ensure the weight variation of animals was minimal and did not exceed ± 20% of the mean weight of each sex.

Identification of the test system

The animals were individually identified by uniquely numbered electronic microchip. Cages were appropriately identified (using a colour-coded procedure) with study information including study number, study type, start date, number and sex of animals, together with a description of the dose level and proposed time of necropsy.

Experimental observations

Health monitoring

All animals were examined at the beginning and the end (nominal) of the working day to ensure that they were in good health and displayed no signs of overt toxicity. Any animal that showed marked signs of ill health was isolated and killed as described below. All decedents were subjected to necropsy and findings were recorded in the raw data but have not been reported.

Post dosing observations

An individual record was maintained of the clinical condition of all Range-Finder Experiment and Main Experiment animals dosed in the study. Satellite animals were for blood sampling purposes only and were monitored but no individual records of clinical conditions were made.

Observation times were as follows:

Table 5: Post dosing observation times

Animals	Day	Approximate observation time
Range-Finder Experiment	1	Immediate, 0.5, 1.0, 2.0 and 4.0-6.0 hours post dose
	2 to 7	Pre-dose, immediate, 0.5, 1.0, 2.0 and 4.0-6.0 hours post dose
Main Experiment	1	Immediate, 1.0, 2.0 and 4.0 hours post dose
	2 to 7	Pre-dose, immediate, 1.0, 2.0 and 4.0 hours post dose
	8-28	Pre-dose, immediate, 2.0 and 4.0 hours post dose
	29-31	Once daily

Body weights

Individual body weights were recorded as follows:

Table 6: Body weight recording

Animals	Day body weight was recorded
Range-Finder Experiment	Day 1 (prior to dosing) and Day 7 (prior to termination)
Main Experiment	Day -1 (at study set-up), Day 1, 8, 15, 22 (prior to dosing), Day 29 [a] and Day 31 (prior to necropsy)
Decedents	Prior to necropsy
Satellites	Day -1 (at study set-up), Day 1, 8, 15, 22 (prior to dosing)

[a] See minor deviations from protocol, Appendix 4

Data for satellite animals have not been reported.

Food and water consumption

Food and water consumption were recorded weekly for the Main Experiment. As data for a full week were not obtained for the Range-Finder Experiment these data have not been reported. These were calculated and reported as g food consumed/animal/week or mL water consumed/animal/week respectively.

Bioanalysis blood sampling procedures

Blood samples were taken at the following time points:

Table 7: Satellite animal bleed times

Group	Male	Sample time (hours after Day 28 administration)
1	101-103	2 [b]
4	104-106	0.5 [a], 4 [b]
4	107-109	1 [a], 8 [b]
4	110-112	2 [a], 24 [b]

[a] Orbital sinus bleed, under isofluorane anaesthesia.
[b] Cardiac puncture bleed, under terminal isofluorane anaesthesia.

Approximately 0.5 mL of blood was collected at room temperature into lithium heparin tubes and mixed thoroughly before placing in a cooled kyrorack. Following centrifugation (2300 *g*, 4°C, 10 minutes) plasma was separated into labelled polypropylene tubes and stored frozen at <-50°C.

Peripheral blood sampling for micronucleus analysis

Blood was taken via the tail vein on Day -1 and Day 4 or via cardiac puncture on Day 31 (at necropsy whilst under terminal isofluorane anaesthesia) from all animals in Groups 1-4.

In-life bleeds were performed by drawing approximately 0.1 mL of blood from the tail vein into capillary tubes pre-filled with K_2EDTA anticoagulant solution. The capillary tubes were emptied into microfuge tubes containing 0.35 mL K_2EDTA solution and the microfuge tubes then inverted several times and stored at room temperature prior to fixing .

Terminal bleeds were performed by removing 0.5-1 mL of whole blood into K_2EDTA tubes and mixed gently for at least two minutes on an automatic mixer. Blood samples in anticoagulant were placed on water-ice until required. 0.1 mL of each K_2EDTA blood sample was added to microfuge tubes containing 0.35 mL K_2EDTA solution and the microfuge tubes then inverted several times and stored at room temperature prior to fixing .

0.18 mL of the blood/anticoagulant mix was added to chilled tubes containing blood fixative (methanol) and vortex mixed. Once fixed, blood samples were stored at <-50°C.

Animal euthanasia and necropsy

Moribund animals were killed by an overdose of sodium pentobarbitone, given via intraperitoneal injection and subsequently ensured by cervical dislocation. Necropsy of all decedents was performed and macro observations recorded to determine possible cause of morbidity/death.

Range-Finder Experiment animals were killed by an overdose of sodium pentobarbitone, given via intraperitoneal injection and subsequently ensured by cervical dislocation. No necropsy was performed.

Main Experiment animals were killed by terminal anaesthesia with isofluorane subsequently ensured by exsanguination. Animals were necropsied in ascending group order.

Satellite animals were killed and discarded post final sample occasion. Animals were not allowed to recover from the isofluorane anaesthesia and death was confirmed by cervical dislocation.

Tissue samples

No tissues were taken from Range-Finder Experiment animals. The following samples were retained from Mutation Experiment animals.

Table 8: Tissue samples

Group	Tissues for mutation analysis	Reserve samples for possible future mutation analysis
1	L, D	Sp (vd), GC (st)
2	L, D	Sp (vd), GC (st)
3	L, D	Sp (vd), GC (st)
4	L, D	Sp (vd), GC (st)

L	Liver
D	Duodenum
Sp (vd)	Spermatazoa from vas deferens
GC (st)	Developing germ cells from seminiferous tubules

Liver and duodenum weights were recorded and the tissues rinsed in phosphate buffered saline (PBS) prior to flash freezing in liquid nitrogen. Frozen samples were moved to a freezer at <-50°C for long term storage.

DNA extraction

DNA from the liver and duodenum was extracted using the Agilent RecoverEase™ system (Agilent Technologies UK Ltd, Stockport, UK) according to the manufacturer's instructions. Prior to extraction the tissues were homogenised as follows: a slice of liver or duodenum was excised from the frozen tissue, thawed and homogenised in 5 mL lysis buffer using a Wheaton Douce homogeniser.

Isolated genomic DNA from all tissues was stored at 2-8°C until required for packaging.

Culturing of bacteria for transfection

An overnight (approximately16 hours) culture of *E. coli* C *lac*$^-$ *galE*$^-$, inoculated from a frozen stock, was grown in a shaking incubator at 37±1°C in 10 mL of Luria broth (LB) containing maltose (0.2% w/v), *Kanamycin* (50 µg/mL) and *Ampicillin* (50 µg/mL). An aliquot (1 mL) of this overnight culture was inoculated into fresh LB medium (98 mL) together with 1 mL of 20% w/v maltose and incubated with shaking at 37±1°C for approximately 4-5 hours. The cells were centrifuged (approximately 2020 x 'g') for 10 minutes and resuspended in approximately 80 mL of LB medium containing 10 mM magnesium sulphate. The cell suspension was stored on ice until used for phage adsorption.

DNA packaging into bacteriophage heads

For each tissue/animal, approximately 5 µL of DNA solution (containing about 7.5 µg of DNA for maximum packaging efficiency) were mixed with Agilent Transpack packaging reagents (Agilent Technologies UK Ltd, Stockport, UK) according to the manufacturer's instructions. Following the necessary incubation and mixing procedures, the resulting mixture contained DNA packaged into bacteriophage heads ready for transfection.

Transfection of host bacteria

The assay involved the scoring of titration plates to determine the total number of plaque-forming units (pfu), and selection plates using the positive selection method to determine the numbers of mutants.

Titration plates

For the titration plates, packaged DNA (10 µL) was diluted with SM buffer (190 µL) and 10 µL of this dilution adsorbed to 500 µl suspension of *E. coli* C *lac*$^-$ *gal*E$^-$ Kan^r (*gal*E$^-$ Amp^r) for approximately 20-30 minutes at room temperature. After adsorption, the phage/bacteria was suspended in 12 mL 1:3 LB:NaCl, 0.75% w/v agar (top agar) containing 10 mM magnesium sulphate and plated onto petri dishes (14 cm diameter) containing 12 mL 1:3 LB:NaCl, 1.5% w/v agar (bottom agar). Once the agar had gelled, the plates were inverted and incubated overnight at 37±1°C.

Positive selection plates

The remaining packaged DNA was divided approximately equally into 3 tubes and incubated at room temperature with bacterial suspension (500 µL/tube) for approximately 20-30 minutes, suspended in top agar (as above) containing 10 mM magnesium sulphate and 0.3% w/v phenyl-galactose (P-gal), poured onto plates containing bottom agar (as above) and incubated overnight at 37±1°C.

Scoring of plates
Following overnight incubation, plates were either scored immediately or stored at 2-8°C and scored as soon as possible.

The number of clear plaques on each plate were counted manually and recorded in the raw data. This data was used to determine the number of plaque forming units (pfu) per reaction.

Packaging and transfection controls
Tissues were processed and analysed using a 'block' design, where DNA samples from the negative and positive control groups and each treatment group were processed together. As data sets for each tissue were nearing completion it became impractical to always have animals from each group in any one set of reactions, however, tissue specific positive control DNA were always included in each set, to confirm the success of each packaging and plating occasion. Data from a packaging occasion were only accepted if the concurrent positive control DNA packaging reaction yielded a characteristic elevated mutant frequency compared to historical negative control mutant frequencies.

Flow cytometry analysis of peripheral blood for micronucleus assessments
Fixed blood samples were thawed and washed with pre-chilled Hanks buffer and isolated by centrifugation (1376 rpm, 5 minutes).Cell pellets were stored at 2-8°C or on ice until staining.

Washed samples were incubated with RNase and two fluorescently labelled antibodies:

1. Anti-CD71-FITC which binds to the transferrin receptor (thereby staining the target reticulocytes population).

2. Anti-CD61-PE which binds to platelets.

Following incubation cells were stored at 2-8°C until analysis.

Prior to analysis the DNA stain (propidium iodide) was added to each sample. These staining steps were performed according to the kit manufacturer's recommendations and Covance SOP.

Blood analysis was conducted using the MicroFlow® method and gating requirements as specified by the kit manufacturer and Covance SOP.

Where possible, 20000 reticulocytes were analysed from each blood sample. To identify each type of cell, fluorescent colour was used. Red indicated that a micronuclei was present (propidium iodide-stained DNA). Reticulocytes (highly stained) or mature erythrocytes (weakly stained) showed up as green (CD71-FITC antibody), and platelets, which were excluded from analysis, showed up as yellow (CD61-PE antibody). The frequency of high CD71 positive reticulocytes (%RET) among total red blood cells (RBC) was determined as the toxicity measure. The number of mature erythrocytes (NCE), micronucleated mature erythrocytes (MN NCE), reticulocytes and micronucleated reticulocytes (MN RET) were measured per sample.

Analysis of results
The unit of analysis was the animal.

The total number of mutant plaques and the total (titre) number of pfu are reported for the liver and duodenum for each animal. The mutant frequency (MF) was calculated for each animal and, from these figures, the mean MF per treatment group. The data obtained with the liver in the treatment groups were compared to the relevant concurrent control data. No historical control data were available for the duodenum.

Statistical analysis of MF per tissue was performed as follows:

Groups 1, 2, 3, 4 were analysed using one-way analysis of variance (ANOVA). An overall dose response test was performed along with Dunnett's test for pairwise comparisons of each treated group with the vehicle control. All tests were performed with a one-sided risk for increasing response. Levene's test for equality of variances across the groups was also performed and in all cases showed no evidence of heterogeneity ($P>0.01$).

For the micronucleus data the % reticulocytes (RET) for each animal and the mean for each group were calculated and were used as an indication of target cell toxicity. The % micronucleated (MN) RET and % MN mature/normochromatic erythrocytes (NCE) for each animal and the group mean % MN RET (± standard deviation) % MN NCE (± standard deviation) were calculated and were used as an indication of genotoxic potential.

Statistical analysis of micronucleus data was performed as follows:

The percentage of MN RET in each treated group was compared with the vehicle control using Wilcoxon Rank Sum test. The tests were interpreted with one-sided risk for increased frequency with increasing dose. Probability values of $p \leq 0.05$ were accepted as significant. The Terpstra-Jonckheere test for dose response was also be performed. Analysis was performed separately for Day 4 and Day 31 data only. No analysis was performed on Day -1 data. These were used solely as background data for interpretation.

Acceptance criteria

Acceptance criteria for individual packaging reactions

For every packaging reaction the data were accepted as valid and used for mutation assessment if the following criteria were met:

1. The concurrent positive control DNA packaging reaction yielded an elevated mutant frequency compared to historical vehicle control data.

2. The pfu for each sample on any packaging occasion exceeded 30,000, although in the event of a poorly packaging sample data were accepted if the pfu fell between 10,000 and 30,000.

3. Unless pfu numbers were low (<30,000), at least 1 mutant plaque should be present in each sample. Where low pfu numbers or low MF were obtained, nil mutant counts may be accepted, if this result was not considered markedly different to that obtained on other packaging occasions.

Acceptance criteria for the assay

The assay was considered valid if all the following criteria were met:

1. The group mean vehicle control MF was comparable with the laboratory's historical vehicle control ranges (where available), and

2. At least five animals out of each group were available for analysis, and

3. A total of at least 200,000 pfu from at least three packaging occasions were obtained per tissue per animal

4. Acceptable flow cytometry results were obtained for the 3 biological controls.

Acceptance of data that did not meet these criteria are discussed in the Results section.

Evaluation criteria

For valid data, the test article was considered positive if:

1. a statistically significant increase in the MF occurs at one or more dose levels in at least one of the tissues examined, or

2. a statistically significant increase in %MN RET compared to concurrent vehicle control group is observed on either Day 4 or Day 31.

The test article was considered negative in this assay if neither of these criteria were met.

The biological relevance of any positive findings were considered in the context of the laboratory's background control historical database.

Computer Systems

The major computer systems used on this study were as follows:

Table 9: Computer Systems

Activity	Computer System
Scheduling	CMS (Covance Management System)
Formulations	Talisman
In-life data collection	Connex
Sample Management	Nautilus / Clinaxys
Data generation and collation	Costar / Mutamouse.xls / SAS
Report generation	Microsoft Office / Adobe Acrobat

Version numbers of the systems are held on file at Covance.

RESULTS

Range-Finder Experiment

Animal body weights recorded in the Range-Finder Experiment are presented in Table 13 (individual weights) and Table 14 (group mean weights); clinical observations recorded in this experiment are presented in Table 22.

Animals dosed at 200 or 350 mg/kg/day displayed no clinical signs of toxicity during seven days of dosing. No loss of body weight recorded, although animals dosed at 350 mg/kg/day did not gain weight over the dosing period. Animals dosed at 500 or 700 mg/kg/day displayed several marked signs of toxicity, which included hunched posture and decreased activity. On Day 1 of dosing at 700 mg/kg, one male animal was killed *in extremis* due to its poor condition. On Day 2 the remaining animals dosed at 700 mg/kg showed clinical signs of toxicity prior to dosing and therefore administration at this dose level was halted. On Day 3 one male animal dosed at 500 mg/kg/day died and the remaining two animals were killed *in extremis* due to their poor condition. Prior to dosing on Day 4 all female animals dosed at 500 mg/kg/day showed severe signs of toxicity and dosing was halted. Necropsy of all decedents identified no evidence of any mal-dosing events. Weight loss was noted in all animals.

From these data a dose of 350 mg/kg/day was considered to be a suitable estimate of the maximum tolerated dose and consequently was selected as the maximum dose for the Main Experiment. Two lower doses of 120 and 235 mg/kg/day were also selected for testing.

No substantial difference in toxicity was observed between males and females in the Range-Finder, therefore male animals only were used in the Main Experiment.

Mutation Experiment

Body weights

Individual animal body weights are presented in Table 15. No clearly treatment-related loss of body weight or reduced body weight gain was observed over the dosing period (Table 16 and Table 17).

Food and water consumption

Food and water consumptions are presented in Table 20 and Table 21 respectively. In week 1 reduced food consumption was observed solely at the high dose group, however from week 2 onwards food consumption for this group was similar to the

vehicle control. No differences were observed between the vehicle control and the low or intermediate dose groups.

Clinical signs

Clinical observations recorded in this experiment are presented in Table 23.

No clinical signs of toxicity were observed in any animal following treatments with vehicle or 3-acetyl-2,5-dimethylthiophene at doses of 120 or 235 mg/kg/day.

Dosing with 3-acetyl-2,5-dimethylthiophene at 350 mg/kg/day induced signs of piloerection and hunched posture in all Main Experiment animals on Day 2. Due to its poor condition, animal 20 was not dosed on Day 3 or 4. Although the remaining animals were in better health it was considered prudent to reduce the dose level to 300 mg/kg/day, given that a further 26 days of dosing were required. Hunched posture and/or piloerection were observed in all animals, with a few other signs also apparent (see Table 23) on Days 3-5. From Day 6 onwards no further clinical signs of toxicity were recorded. The only exception to this was animal 23: on Day 7 several clinical signs were observed and this animal was subsequently killed *in extremis* due to its poor condition. Necropsy of this decedent identified no evidence of any mal-dosing event.

Validity of study

The majority of data from any individual packaging reaction resulted in at least 30,000 pfu. A limited number of packaging reactions resulted in <30,000 pfu, but in all cases these were >20,000 pfu and were consistent with other packaging data generated for the sample. Consequently, acceptance of these data was considered justified.

For all animals data were generated for at least 200,000 pfu per tissue, generated from at least three independent packaging reactions.

For each set of data accepted for mutation assessment, concurrent packaging of at least one positive control DNA sample yielded a characteristic elevated mutant frequency compared to historical negative control mutant frequencies; this confirming the correct functioning of the packaging reactions on each occasion.

At least 1 million pfu were obtained per group, per tissue from a minimum of five animals.

Group mean vehicle control mutant frequency data for liver were comparable with the laboratory's historical data and the duodenum vehicle control data were considered comparable with published literature for GI tract (Lambert *et al.*, 2005).

Acceptable flow cytometry results were obtained for the three biological controls.

The study was therefore accepted as valid.

Analysis of mutation data

Following administration of 120, 235 or 350/300 mg/kg/day of 3-acetyl-2,5-dimethylthiophene the following group mutant frequency data were obtained:

Liver analysis

A statistically significant increase in mutant frequency (MF) was observed in the liver following dosing with 3-acetyl-2,5-dimethylthiophene at 235 and 350/300 mg/kg/day (Table 10). Although the low dose of 3-acetyl-2,5-dimethylthiophene did not induce a significant increase in MF, a small increase in group mean MF compared to the concurrent vehicle control was apparent and a significant linear trend was also observed.

Qualitative analysis of individual animal MF showed that all animals dosed at 235 and 350/300 mg/kg/day had MF that exceeded the concurrent vehicle controls (Table 25) and also the mean and median values of the laboratory's historical control data (Appendix 1).

The data confirmed that 3-acetyl-2,5-dimethylthiophene did induce mutation in the liver of treated animals.

Table 10: Mutant frequency group summary – Liver

Group	Treatment	Dose (mg/kg/day)	Group Mean MF (x 10^{-6} ± SD)
1	Vehicle control	0	33.25 ± 11.41
2	3-acetyl-2,5-dimethylthiophene	120	52.76 ± 13.35
3	3-acetyl-2,5-dimethylthiophene	235	97.23 ± 21.43 **
4	3-acetyl-2,5-dimethylthiophene	350/300	139.23 ± 53.79 *** A, DR***

** P≤0 01
*** P≤0.001
A ANOVA, dose response and Dunnett's (Groups 2, 3 and 4 vs Group 1)
DR Dose response
DR Significant dose response test

Duodenum analysis

No statistically significant increases in mutant frequency (MF) or significant linear trend were observed in the duodenum following dosing with 3-acetyl-2,5-dimethylthiophene at 120, 235 or 350/300 mg/kg/day (Table 11). Qualitative analysis of individual animal MF showed that whilst some animals dosed with 3-acetyl-2,5-dimethylthiophene had MF that exceeded the vehicle control group, the majority were comparable with the concurrent vehicle controls (Table 26).

The data confirmed that 3-acetyl-2,5-dimethylthiophene did not induce mutation in the duodenum.

Table 11: Mutant frequency group summary – Duodenum

Group	Treatment	Dose (mg/kg/day)	Group Mean MF (x 10^{-6} ± SD)
1	Vehicle control	0	71.11 ± 7.51
2	3-acetyl-2,5-dimethylthiophene	120	103.09 ± 47.32
3	3-acetyl-2,5-dimethylthiophene	235	111.24 ± 39.28
4	3-acetyl-2,5-dimethylthiophene	350/300	93.18 ± 30.15 A

A ANOVA, dose response and Dunnett's (Groups 2, 3 and 4 vs Group 1)

Analysis of micronucleus data

The data in Table 12 confirmed there was no evidence of treatment-related toxicity to the peripheral blood in any 3-acetyl-2,5-dimethylthiophene-treated groups; the frequency of reticulocytes (%RET) in all 3-acetyl-2,5-dimethylthiophene-treated groups was comparable to the concurrent vehicle controls on both Day 4 and Day 31.

Small but statistically significant increases in micronuclei (Wilcoxon Rank Sum Test at the 5% level) were observed in micronucleus frequencies following dosing with 3-acetyl-2,5-dimethylthiophene at 350/300 mg/kg/day on Day 4 and at 235 mg/kg/day on Day 31. There was no significant linear trend in either data set. Although these small increases in group mean MN RET frequency were observed (compared to the concurrent vehicle control) this was considered to be within the natural variation of the assay. Furthermore, the group mean MN-RET for the high dose animals (350/300 mg/kg/day 3-acetyl-2,5-dimethylthiophene) on Day 4 and the intermediate dose animals (235 mg/kg/day 3-acetyl-2,5-dimethylthiophene) on Day 31 were comparable with the base line group mean for these groups obtained on Day 1, confirming that this result was of no biological relevance.

The data confirmed that 3-acetyl-2,5-dimethylthiophene did not induce micronuclei in peripheral blood after either 4 days or 28 days of dosing.

Table 12: Micronucleus frequency group summary

Sample Day	Group / Treatment (mg/kg/day)	%RET Mean	%RET SD	%MN-NCE Mean	%MN-NCE SD	%MN-RET Mean	%MN-RET SD
Day-1	1 / Vehicle control (0)	2.30	0.34	0.277	0.033	0.48	0.05
	2 / 3-acetyl-2,5-dimethylthiophene (120)	2.09	0.19	0.287	0.030	0.49	0.04
	3 / 3-acetyl-2,5-dimethylthiophene (235)	2.38	0.49	0.279	0.026	0.47	0.03
	4 / 3-acetyl-2,5-dimethylthiophene (350/300)	2.81	0.28	0.300	0.013	0.50	0.03
Day 4	1 / Vehicle control (0)	2.02	0.42	0.241	0.013	0.35	0.03
	2 / 3-acetyl-2,5-dimethylthiophene (120)	1.79	0.27	0.242	0.014	0.38 NS	0.07
	3 / 3-acetyl-2,5-dimethylthiophene (235)	1.71	0.37	0.250	0.019	0.32 NS	0.07
	4 / 3-acetyl-2,5-dimethylthiophene (350/300)	1.70	0.46	0.264	0.010	0.57 P≤0.05	0.22
Day 31	1 / Vehicle control (0)	1.49	0.25	0.241	0.016	0.37	0.05
	2 / 3-acetyl-2,5-dimethylthiophene (120)	1.84	0.21	0.262	0.008	0.42 NS	0.05
	3 / 3-acetyl-2,5-dimethylthiophene (235)	1.57	0.28	0.267	0.007	0.42 P≤0.05	0.04
	4 / 3-acetyl-2,5-dimethylthiophene (350/300)	1.56	0.20	0.286	0.014	0.40 NS	0.06
						W	

Terpstra-Jonckheere trend test NS
NS Not significant
W Wilcoxon Rank Sum Test

CONCLUSION

It was concluded that 3-acetyl-2,5-dimethylthiophene induced mutation in the *lacZ* transgene in the liver of male Muta™Mice that had been dosed daily for 28 days at up to 300 mg/kg/day (an estimate of the maximum tolerated dose). 3-acetyl-2,5-dimethylthiophene did not induce mutation in the duodenum of the same animals, nor did it induce an increase in micronucleated reticulocytes in the peripheral blood.

REFERENCES

Ashby, J and Tinwell, H (1994) Use of transgenic mouse lacI/Z mutation assays in genetic toxicology. Mutagenesis, 9 (3), 179-181.

Committee on Mutagenicity of chemicals in food, consumer products and the environment (COM). Guidance on a strategy for testing of chemicals for mutagenicity (2000).

Dean, S W, Brooks, T M, Burlinson, B, Mirsalis, J, Myhr, B, Recio, L and Thybaud, V (1999) Transgenic mouse mutation assay systems can play an important role in regulatory mutagenicity testing *in vivo* for the detection of site-of-contact mutagens. Mutagenesis, 14 (1), 141-151.

Dean, S W and Myhr, B (1994) Measurement of gene mutation *in vivo* using Muta^{TM}Mouse and positive selection for LacZ$^-$ phage. Mutagenesis, 9 (3), 183-185.

Fielder R J, Allen J A, Boobis A R, Botham P A, Doe J, Esdaile D J, Gatehouse D G, Hodson-Walker G, Morton D B, Kirkland D J and Richold M (1993) Report of the BTS/UKEMS Working Group on Dose Setting in *in vivo* Mutagenicity Assays. Human & Experimental Toxicology 12, 189-198.

Gossen J A, de Leeuw W J, Tan C H, Zwarthoff E C, Berends F, Lohman P H, Knook D L, Vijg J. (1989). Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*, Proceedings of the National Academy of Science USA 86, 7971-5.

Gossen, J and Vijg, J (1993) A selective system for LacZ$^-$ phage using a galactose-sensitive *E. coli* host. Biotechniques, 14, 326-330.

Heddle, J A, Shaver-Walker, P, Tao, K S and Zhang, X B (1995) Treatment protocols for transgenic mutation assays *in vivo*. Mutagenesis, 10 (6), 467-470.

Lambert I B, Singer T M, Boucher S E, Douglas G R (2005). Detailed review of transgenic rodent mutation assays. Mutation Research 590, 1-280.

OECD (2011). "Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays". In OECD Guidelines for the testing of chemicals. OECD Paris, Test Guideline 488.

Tennant, R W, Hansen L and Spalding J (1994) Gene manipulation and genetic toxicology. Mutagenesis, 9 (3), 171-174.

Thybaud V, Dean S, Nohmi T, de Boer J, Douglas G R, Glickman B W, Gorelick N J, Heddle J A, Heflich R H, Lambert I, Martus H-J, Mirsalis J, Suzuki T, Yajima N (2003) Reports of the 3rd International Workshop on Genotoxicity Testing (IWGT), 2002, Plymouth, UK. *in vivo* transgenic mutation assays. Mutation Research 540, 141-151.

TABLES – ANIMAL DATA

Table 13: Range-Finder individual body weights

Test Article: 3-acetyl-2,5-dimethylthiophene
Group: 1
Level (mg/kg/day): 200

Group/ Sex	Animal Number	Individual body weights (g) for Day: 1	7
1M	201	25.6	26.5
1M	202	24.9	26.4
1M	203	23.2	25.4
1F	204	19.7	20.4
1F	205	19.7	20.5
1F	206	20.0	20.5

Test Article: 3-acetyl-2,5-dimethylthiophene
Group: 2
Level (mg/kg/day): 700

Group/ Sex	Animal Number	Individual body weights (g) for Day: 1	2
2M	207	25.1	22.9
2M	208	24.3	21.8
2M	209	25.0	-
2F	210	21.3	19.1
2F	211	21.2	18.9
2F	212	21.6	20.1

Test Article: 3-acetyl-2,5-dimethylthiophene
Group: 3
Level (mg/kg/day): 350

Group/ Sex	Animal Number	Individual body weights (g) for Day: 1	7
3M	213	28.7	29.2
3M	214	26.4	25.9
3M	215	27.5	27.7
3F	216	20.1	20.6
3F	217	22.1	20.9
3F	218	20.9	21.8

Test Article: 3-acetyl-2,5-dimethylthiophene
Group: 4
Level (mg/kg/day): 500

Group/ Sex	Animal Number	Individual body weights (g) for Day: 1	3
4M	219	26.4	23.5
4M	220	24.7	22.0
4M	221	28.1	24.9
4F	222	18.9	NT
4F	223	20.0	NT
4F	224	18.7	NT

NT = Not Taken

Table 14: Range-Finder group mean body weights

Test Article	3-acetyl-2,5-dimethylthiophene
Group	1
Level (mg/kg/day)	200

Day		Mean body weights (g) for Group: 1M
1	Mean	24.6
	SD	1.23
7	Mean	26.1
	SD	0.61

Day		Mean body weights (g) for Group: 1F
1	Mean	19.8
	SD	0.17
7	Mean	20.5
	SD	0.06

Test Article	3-acetyl-2,5-dimethylthiophene
Group	2
Level (mg/kg/day)	700

Day		Mean body weights (g) for Group: 2M
1	Mean	24.8
	SD	0.44
2	Mean	22.4
	SD	0.78

Day		Mean body weights (g) for Group: 2F
1	Mean	21.4
	SD	0.21
2	Mean	19.4
	SD	0.64

Test Article	3-acetyl-2,5-dimethylthiophene
Group	3
Level (mg/kg/day)	350

Day		Mean body weights (g) for Group: 3M
1	Mean	27.5
	SD	1.15
7	Mean	27.6
	SD	1.65

Day		Mean body weights (g) for Group: 3F
1	Mean	21.0
	SD	1.01
7	Mean	21.1
	SD	0.62

Test Article	3-acetyl-2,5-dimethylthiophene
Group	4
Level (mg/kg/day)	500

Day		Mean body weights (g) for Group: 4M
1	Mean	26.4
	SD	1.70
3	Mean	23.5
	SD	1.45

Day		Mean body weights (g) for Group: 4F
1	Mean	19.2
	SD	0.70
3	Mean	-
	SD	-

Table 15: Main Experiment individual body weights

Test Article	Vehicle	3-acetyl-2,5-dimethylthiophene		
Group	1	2	3	4
Level (mg/kg/day)	0	120	235	350/300

Group/ Sex	Animal Number	Individual body weights (g) for Day: -1	1	7	8	15	22
1M	1	23.2	23.0	NT	22.8	23.5	22.5
1M	2	24.2	24.5	NT	25.1	26.3	25.6
1M	3	25.1	24.6	NT	24.8	25.0	24.6
1M	4	27.0	26.7	NT	27.5	28.7	28.4
1M	5	23.1	23.0	NT	23.8	26.0	24.7
1M	6	28.5	27.8	NT	28.4	28.1	27.2
2M	7	25.7	25.3	NT	26.0	25.9	26.1
2M	8	26.1	25.4	NT	26.6	26.3	26.0
2M	9	26.1	25.6	NT	26.5	27.2	26.4
2M	10	23.8	23.1	NT	23.6	24.6	24.6
2M	11	25.8	25.4	NT	25.7	27.1	26.2
2M	12	25.3	24.9	NT	26.4	27.9	26.8
3M	13	25.5	24.7	NT	25.5	27.3	26.7
3M	14	25.7	24.8	NT	25.5	26.1	25.5
3M	15	26.2	25.8	NT	26.6	27.0	27.4
3M	16	26.2	25.7	NT	26.9	28.1	28.0
3M	17	22.4	22.2	NT	23.1	23.7	23.3
3M	18	24.4	23.7	NT	25.4	26.3	25.7
4M	19	24.4	24.1	NT	23.5	27.0	28.2
4M	20	23.9	24.1	NT	24.6	26.5	26.3
4M	21	25.5	24.7	NT	27.3	30.0	29.4
4M	22	27.5	26.2	NT	26.0	30.0	29.8
4M	23	24.6	24.8	18.5	-	-	-
4M	24	24.7	25.0	NT	26.4	28.1	26.5

NT = Not Taken

Test Article	Vehicle	3-acetyl-2,5-dimethylthiophene		
Group	1	2	3	4
Level (mg/kg/day)	0	120	235	350/300

Group/ Sex	Animal Number	Individual body weights (g) for Day: 29	31
1M	1	23.8	23.6
1M	2	26.0	26.3
1M	3	25.4	25.9
1M	4	29.4	29.6
1M	5	24.7	25.2
1M	6	28.0	28.3
2M	7	26.7	26.5
2M	8	27.7	27.6
2M	9	27.5	27.6
2M	10	25.4	24.8
2M	11	26.9	26.1
2M	12	28.3	27.7
3M	13	26.1	26.5
3M	14	26.1	26.0
3M	15	27.8	27.2
3M	16	28.0	28.6
3M	17	24.1	23.7
3M	18	25.5	25.8
4M	19	28.7	27.6
4M	20	27.1	26.5
4M	21	29.6	29.2
4M	22	30.7	30.9
4M	23	-	-
4M	24	27.6	26.8

Table 16: Main Experiment group mean body weights

Test Article		Vehicle	3-acetyl-2,5-dimethylthiophene		
Group		1	2	3	4
Level (mg/kg/day)		0	120	235	350/300

		Mean body weights (g) for Group:			
Day		1M	2M	3M	4M
-1	Mean	25.2	25.5	25.1	25.1
	SD	2.17	0.87	1.46	1.29
1	Mean	24.9	25.0	24.5	24.8
	SD	1.96	0.94	1.36	0.77
7	Mean	-	-	-	18.5
	SD	-	-	-	-
8	Mean	25.4	25.8	25.5	25.6
	SD	2.15	1.13	1.34	1.51
15	Mean	26.3	26.5	26.4	28.3
	SD	1.93	1.17	1.51	1.64
22	Mean	25.5	26.0	26.1	28.0
	SD	2.09	0.75	1.67	1.61
29	Mean	26.2	27.1	26.3	28.7
	SD	2.11	1.00	1.46	1.46
31	Mean	26.5	26.7	26.3	28.2
	SD	2.16	1.15	1.63	1.84

Table 17: Main Experiment group mean body weight gain

Test Article		Vehicle	3-acetyl-2,5-dimethylthiophene		
Group		1	2	3	4
Level (mg/kg/day)		0	120	235	350/300

		Mean body weight gain (g) for Group:			
Day		1M	2M	3M	4M
-1-1	Mean	-0.3	-0.5	-0.6	-0.3
	SD	0.34	0.15	0.26	0.65
1-7	Mean	-	-	-	-6.3
	SD	-	-	-	-
8-15	Mean	0.9	0.7	0.9	2.8
	SD	0.88	0.76	0.52	0.99
15-22	Mean	-0.8	-0.5	-0.3	-0.3
	SD	0.38	0.53	0.40	1.01
22-29	Mean	0.7	1.1	0.2	0.7
	SD	0.46	0.45	0.53	0.35
29-31	Mean	0.3	-0.4	0.0	-0.5
	SD	0.26	0.35	0.48	0.49

Food and water consumption data

Table 18: Range-Finder Experiment group mean weekly food consumption

Test Article		3-acetyl-2,5-dimethylthiophene			
Group		1	2	3	4
Level (mg/kg/day)		200	700	350	200

		Mean food consumption (g/animal/period) for Group:			
Day		1M	2M	3M	4M
1-6	Mean	18.7	-	31.5	-
Day		1F	2F	3F	4F
1-6	Mean	16.1	-	28.7	-

Table 19: Range-Finder Experiment group mean weekly water consumption

Test Article		3-acetyl-2,5-dimethylthiophene			
Group		1	2	3	4
Level (mg/kg/day)		200	700	350	200

		Mean water consumption (g/animal/period) for Group:			
Day		1M	2M	3M	4M
1-6	Mean	20.3	-	27.3	-
Day		1F	2F	3F	4F
1-6	Mean	22.4	-	21.2	-

Table 20: Main Experiment group mean weekly food consumption

Test Article	Vehicle	3-acetyl-2,5-dimethylthiophene		
Group	1	2	3	4
Level (mg/kg/day)	0	120	235	350/300

		Mean food consumption (g/animal/week) for Group:			
Week		1M	2M	3M	4M
1	Mean	22.0	20.9	21.2	13.9
	SD	0.75	0.02	0.59	3.47
2	Mean	20.5	20.5	20.9	24.8
	SD	1.34	1.98	0.05	1.28
3	Mean	19.8	21.3	21.9	25.0
	SD	0.94	0.97	0.47	2.26
4	Mean	22.0	21.8	21.5	23.9
	SD	0.52	1.08	0.33	2.14

Table 21: Main Experiment group mean weekly water consumption

Test Article	Vehicle	3-acetyl-2,5-dimethylthiophene		
Group	1	2	3	4
Level (mg/kg/day)	0	120	235	350/300

Week		Mean water consumption (g/animal/week) for Group: 1M	2M	3M	4M
1	Mean	24.0	25.5	29.6	24.0
	SD	4.69	2.59	1.13	3.33
2	Mean	27.2	24.5	31.4	34.9
	SD	0.19	5.09	2.83	7.59
3	Mean	25.2	27.1	31.6	34.0
	SD	2.78	3.79	2.26	9.59
4	Mean	26.2	27.3	30.8	31.6
	SD	1.65	4.53	1.25	6.72

Table 22: Range-finder Experiment clinical observations

Group/ Treatment (mg/kg/day)	Animal number and sex	Clinical Sign										
		Day 1 (hours after administration)					Day 2 (hours after administration)					
		Imm	0.5	1	2	4-6	Pre-dose	Imm	0.5	1	2	4-6
2RF/ 3-acetyl-2,5-dimethylthiophene (700)	207M	√	√	√	PC[1]	√	HP	[a]	-	-	-	-
	208M	√	√	√	PC[1]	√	HP	[a]	-	-	-	-
	209M	√	√	√	PC[1], HP, PI, DA	PC, HP, DA[a]	-	-	-	-	-	-
	210F	√	√	√	PC[1]	√	HP	[a]	-	-	-	-
	211F	√	√	√	PC[1]	PC, HP, DA	HP, PC, DA	[a]	-	-	-	
	212F	√	√	√	PC[1]	√	HP, PC, DA	[a]	-	-	-	-
4RF / 3-acetyl-2,5-dimethylthiophene (500)	219M	√	√	√	√	√	√	√	√	√	√	√
	220M	√	√	√	√	√	√	√	√	DA, PT	DA, PT, PI	DA, PI, PT
	221M	√	√	√	√	√	√	√	√	√	√	√
	222F	√	√	√	√	√	√	√	√	√	√	√
	223F	√	√	√	√	√	√	√	√	√	√	√
	224F	√	√	√	√	√	√	√	√	√	√	√

Group/ Treatment (mg/kg/day)	Animal number and sex	Clinical Sign							
		Day 3 (hours after administration)							Day 4
		Pre-dose	Imm	0.5	1	2	4-6	9	Pre-dose
4RF / 3-acetyl-2,5-dimethylthiophene (500)	219M	PI, PT, DA	PI, HP, PT, DA	HP, PT, PI	DA, HP, PT, PI	HP, DA, PI, PT	LI, PN, TM, BB, LA, PL, HT, PT [a]	-	-
	220M	PI, PT, DA	PI, HP, PT, DA	HP, PT, PI	DA, HP, PT, LA, PI	HP, DA, PI, PT, LA	UB, LI, PN, TM, LA, PL, HT, PT[a]	-	-
	221M	PI, PT, DA	PI, HP, PT, DA	HP, PT, PI, DA	DA, HP, PT, PI	PN, DA, PI, PT, LA, BD	[b]	-	-
	222F	PI	PI	PI	HP, DA, PT, PI	HP, DA, PT, PI	PI, HP, PT, DA	DA, HP, PT, PI	DA, HP, PT, PI
	223F	PI	PI	PI	HP, DA, PT, PI	PT,PI	PI, HP, PT, DA	DA, HP, PT, PI	DA, HP, PT, PI
	224F	PI	PI	PI, DA, PT	HP, DA, PI, PT	HP, DA, PT, PI	PI, HP, PT, DA	DA, HP, PT, PI	DA, HP, PT, PI

No clinical signs observed at 200 or 350 mg/kg/day.

See Table 24 for key to observations.

Table 23: Mutation Experiment clinical Observations

Group/ Treatment (mg/kg/day)	Animal number and sex	Clinical Sign								
		Day 1 (hours after administration)				Day 2 (hours after administration)				
		Imm	1	2	4	Pre-dose	Imm	1	2	4
4 / 3-acetyl-2,5 -dimethylthiophene (350)	19M	√	√	√	√	√	√	PI, HP	PI, HP	PI, HP
	20M	√	√	√	√	√	√	PI, HP	PI, HP	PI, HP
	21M	√	√	√	√	√	√	PI, HP	PI, HP	PI, HP
	22M	√	√	√	√	√	√	PI	PI, HP	PI, HP
	23M	√	√	√	√	√	√	PI, HP	PI, HP	PI, HP
	24M	√	√	√	√	√	√	PI	PI	PI, HP

Group/ Treatment (mg/kg/day)	Animal number and sex	Clinical Sign									
		Day 3 (hours after administration)					Day 4 (hours after administration)				
		Pre-dose	Imm	1	2	4	Pre-dose	Imm	1	2	4
4 / 3-acetyl-2,5 -dimethylthiophene (300)	19M	PI, HP	√	PI	PI, PT, HP	PI, PT, HP, LA	√	√	PI	PI	PI
	20M	PI, HP, LA, PT	ND	ND	ND	ND	PI, HP, DA, PT, LA	ND	ND	ND	ND
	21M	√	√	PI	PI, HP	PI, HP	√	√	PI	PI	PI
	22M	√	√	√	PI, HP	PI, HP	√	√	PI	PI	PI
	23M	PI, HP	√	√	PI, PT, LA, HP	PI, HP	√	√	PI	PI	PI
	24M	√	√	√	PI, PT, LA, HP	PI, HP	√	√	PI	PI	PI

Table continued overleaf.

Table 22 continued: Mutation Experiment clinical Observations

Group/ Treatment (mg/kg/day)	Animal number and sex	Clinical Sign									
		Day 5 (hours after administration)					Day 6 (hours after administration)				
		Pre-dose	Imm	1	2	4	Pre-dose	Imm	1	2	4
4 / 3-acetyl-2,5 -dimethylthi ophene (300)	19M	√	√	PI	PI	PI	√	√	√	√	√
	20M	√	√	PI	PI	PI	√	√	√	√	√
	21M	√	√	PI	PI	PI	√	√	√	√	√
	22M	√	√	PI	PI	PI	√	√	√	√	√
	23M	√	√	PI	PI	PI	√	√	√	√	√
	24M	√	√	PI	PI	PI	√	√	√	√	√

Group/ Treatment (mg/kg/day)	Animal number and sex	Clinical Sign								
		Day 7 (hours after administration)					Day 8 (hours after administration)			
		Pre-dose	Imm	1	2	4	Pre-dose	Imm	2	4
4 / 3-acetyl-2,5 -dimethylthi ophene (300)	19M	√	√	√	√	√	√	√	√	√
	20M	√	√	√	√	√	√	√	√	√
	21M	√	√	√	√	√	√	√	√	√
	22M	√	√	√	√	√	√	√	√	√
	23M	√	√	LA, PI, BD, SA, HP	PI, HP, DA, BD[a]	-	-	-	-	-
	24M	√	√	√	√	√	√	√	√	√

No clinical signs observed at 120 or 235 mg/kg/day, or at any dose after Day 8.

See Table 24 for key to observations.

Table 24: Key to clinical observations

M	Male
F	Female
Imm	Immediately post-dose
√	No abnormality detected
a	Animal killed *in extremis*
b	Animal found dead
BB	Bradypnoea
BD	Dyspnoea
DA	Decreased activity
HP	Hunched posture
HT	Hypothermia
LA	Increased lachrymation
LI	Limp
ND	Not dosed
PC[1]	Palpebral closure – Eyes semi closed
PC	Palpebral closure
PI	Piloerection
PL	Pallor of eyes
PN	Prone
PT	Ptosis
SA	Increased salivation
TM	Tremors
UB	Haematuria

TABLES – GENOTOXICITY DATA

Table 25: 3-acetyl-2,5-dimethylthiophene Liver Mutant Frequencies

Group / Treatment (mg/kg/day)	Animal	pfu	Mutants	Animal MF ($x10^{-6}$)	Number of packaging occasions
1 / Vehicle (0)	1	455,561	23	51.02	3
	2	587,627	15	25.80	3
	3	258,796	11	42.96	3
	4	981,157	29	29.87	3
	5	925,796	19	20.74	3
	6	450,892	13	29.14	3
2 / 3-acetyl-2,5-dimethylthiophene (120)	7	412,873	18	44.06	3
	8	904,452	43	48.05	4
	9	274,137	12	44.24	3
	10	822,411	37	45.47	3
	11	644,989	50	78.34	3
	12	412,206	23	56.39	4
3 / 3-acetyl-2,5-dimethylthiophene (235)	13	442,888	54	123.22	3
	14	590,295	72	123.27	3
	15	563,615	50	89.65	3
	16	447,557	41	92.58	3
	17	993,830	82	83.38	4
	18	1,006,503	71	71.29	4
4 / 3-acetyl-2,5-dimethylthiophene (350/300)	19	596,298	76	128.80	3
	20	908,454	81	90.11	4
	21	588,961	86	147.57	3
	22	707,020	72	102.92	3
	23	ND	ND	ND	ND
	24	797,732	179	226.77	4
Positive control [a]	29	2,595,297	1726	672.10	11
	31	154,744	152	992.69	1
	33	1,756,211	1129	649.68	11

ND No data, animal died

[a] DNA isolated from tissues taken from animals dosed with ENU at 100 mg/kg/day under Covance study 8259749.

Table 26: 3-acetyl-2,5-dimethylthiophene Duodenum mutant frequencies

Group / Treatment (mg/kg/day)	Animal	pfu	Mutants	Animal MF (x10-6)	Number of packaging occasions
1 / Vehicle (0)	1	689,678	42	61.54	3
	2	246,790	20	81.90	4
	3	498,249	38	77.08	3
	4	200,767	14	70.47	4
	5	514,257	36	70.75	4
	6	233,450	15	64.94	3
2 / 3-acetyl-2,5-dimethylthiophene (120)	7	230,115	13	57.09	3
	8	461,564	31	67.88	3
	9	368,184	37	101.56	3
	10	359,513	67	188.34	3
	11	570,952	48	84.96	3
	12	468,234	55	118.71	3
3 / 3-acetyl-2,5-dimethylthiophene (235)	13	330,165	39	119.38	3
	14	459,563	45	98.96	5
	15	474,904	44	93.63	3
	16	441,554	44	100.70	3
	17	344,839	63	184.63	3
	18	216,108	15	70.15	5
4 / 3-acetyl-2,5-dimethylthiophene (350/300)	19	339,503	48	142.88	3
	20	548,274	45	82.95	3
	21	725,029	67	93.39	3
	22	835,751	51	61.67	5
	23	ND	ND	ND	ND
	24	344,839	29	84.99	3
Positive control [a]	29	1,595,464	5188	3286.21	11
	30	1,582,124	5392	3444.22	10
	31	458,896	1709	3763.65	3
	32	313,490	1892	6099.29	3

ND No data, animal died.

[a] DNA isolated from tissues taken from animals dosed with ENU at 100 mg/kg/day under Covance study 8259749.

Table 27: 3-acetyl-2,5-dimethylthiophene Peripheral blood micronucleus data for Day -1

Group / Treatment (mg/kg/day)	Animal No.	All Events	No. NCE	No. MN-NCE	No. RET	No. MN-RET	%RET	%MN RET
1 / Vehicle	1	701695	674847	2251	19798	101	2.85	0.51
	2	1037467	1007129	2488	19755	89	1.93	0.45
	3	906184	880971	2378	19892	108	2.21	0.54
	4	984182	959195	2347	19918	82	2.04	0.41
	5	889886	863776	2551	19901	102	2.26	0.51
	6	803731	779138	2165	19904	96	2.50	0.48
2 / 3-acetyl-2,5-dimethylthiophene (120)	7	965799	939610	2799	19894	106	2.08	0.53
	8	1161277	1134386	2929	19907	93	1.73	0.47
	9	952026	927339	2442	19911	89	2.11	0.45
	10	895238	870669	2326	19910	90	2.24	0.45
	11	922971	897463	3026	19903	97	2.17	0.49
	12	909075	883864	2687	19895	105	2.21	0.53
3 / 3-acetyl-2,5-dimethylthiophene (235)	13	800537	775905	2441	19900	100	2.51	0.50
	14	1168055	1142669	2764	19910	90	1.72	0.45
	15	1059456	1033454	2964	19899	102	1.89	0.51
	16	783851	759942	2119	19911	89	2.56	0.45
	17	659185	634763	1904	19914	86	3.05	0.43
	18	786680	762539	1977	19905	95	2.55	0.48
4 / 3-acetyl-2,5-dimethylthiophene (350/300)	19	667525	643880	2033	19925	92	3.01	0.46
	20	704986	680387	2112	19907	93	2.85	0.47
	21	701041	677172	1976	19897	103	2.86	0.52
	22	869707	844928	2364	19901	99	2.31	0.50
	23	735173	710649	2193	19893	107	2.73	0.54
	24	644886	621191	1861	19907	97	3.11	0.48

Table 28: 3-acetyl-2,5-dimethylthiophene Peripheral blood micronucleus data for Day 4

Group / Treatment (mg/kg/day)	Animal No.	All Events	No. NCE	No. MN-NCE	No. RET	No. MN-RET	%RET	%MN RET
1 / Vehicle	1	761776	735486	1839	19863	77	2.63	0.39
	2	1197975	1158185	2690	19528	74	1.66	0.38
	3	1280618	1225873	2836	19177	62	1.54	0.32
	4	896404	856743	2002	19341	61	2.21	0.31
	5	888830	858102	2259	19682	68	2.24	0.34
	6	1096550	1055633	2524	19355	71	1.80	0.37
2 / 3-acetyl-2,5-dimethylthiophene (120)	7	942802	906517	2299	19457	64	2.10	0.33
	8	1373491	1328870	3160	19415	93	1.44	0.48
	9	927149	883546	1943	19234	54	2.13	0.28
	10	1213110	1173967	2827	19481	78	1.63	0.40
	11	1186521	1140238	2770	19378	77	1.67	0.40
	12	1131948	1083152	2802	19246	74	1.75	0.38
3 / 3-acetyl-2,5-dimethylthiophene (235)	13	828036	790532	1920	19459	70	2.41	0.36
	14	1370438	1309317	3133	19219	83	1.45	0.43
	15	1468445	1410847	3170	19279	51	1.35	0.26
	16	1188560	1139868	2984	19341	49	1.67	0.25
	17	1129587	1086624	3017	19553	65	1.77	0.33
	18	1249016	1198166	3075	19398	61	1.59	0.31
4 / 3-acetyl-2,5-dimethylthiophene (350/300)	19	1115967	1058314	2931	19173	142	1.79	0.74
	20	941300	893212	2310	19129	169	2.11	0.88
	21	1650099	1595455	4113	19360	68	1.20	0.35
	22	1510286	1457424	4019	19260	119	1.31	0.61
	23	862570	821885	2197	19636	89	2.34	0.45
	24	1377521	1309080	3286	19043	69	1.44	0.36

Table 29: 3-acetyl-2,5-dimethylthiophene Peripheral blood micronucleus data for Day 31

Group / Treatment (mg/kg/day)	Animal No.	All Events	No. NCE	No. MN-NCE	No. RET	No. MN-RET	%RET	%MN RET
1 / Vehicle	1	1202932	1174976	3027	19924	76	1.67	0.38
	2	1545202	1515571	3463	19923	77	1.30	0.39
	3	1346872	1317932	3065	19932	68	1.49	0.34
	4	1484358	1459283	3277	19928	72	1.35	0.36
	5	1618482	1589112	4173	19939	61	1.24	0.31
	6	1058479	1032792	2535	19910	90	1.90	0.45
2 / 3-acetyl-2,5-dimethylthiophene (120)	7	1406436	1377623	3692	19926	74	1.43	0.37
	8	1013245	989625	2464	19924	76	1.98	0.38
	9	1032187	1005833	2615	19924	76	1.94	0.38
	10	1009341	982916	2621	19916	84	1.99	0.42
	11	1070606	1044777	2823	19903	97	1.87	0.49
	12	1106426	1081901	2864	19905	95	1.81	0.48
3 / 3-acetyl-2,5-dimethylthiophene (235)	13	1240133	1210132	3165	19910	91	1.62	0.45
	14	1039177	1012087	2725	19909	91	1.93	0.46
	15	1084666	1057015	2736	19928	72	1.85	0.36
	16	1488745	1460323	3915	19910	90	1.35	0.45
	17	1575677	1548874	4304	19921	79	1.27	0.40
	18	1466337	1436278	3912	19921	79	1.37	0.40
4 / 3-acetyl-2,5-dimethylthiophene (350/300)	19	1218896	1190083	3576	19927	73	1.65	0.37
	20	1416889	1387331	4073	19933	67	1.42	0.34
	21	1531711	1503226	3992	19903	97	1.31	0.49
	22	1238426	1210743	3563	19926	74	1.62	0.37
	23	ND	ND	ND	ND	ND	ND	ND
	24	1110896	1083290	3061	19916	84	1.81	0.42

ND No data, animal died.

APPENDICES

Appendix 1
Historical vehicle control data ranges

Table 30: Muta™ Mouse historical control, male liver data

Tissue	Statistic	Mutant Frequency (MF)
Liver	Number of studies	20
	Number of animals	187
	Mean	66
	SD	37.1
	Median	58
	Observed range	8 to 240
	95% reference range	17 to 160
	95% confidence interval	NR

NR = not reported (data not normally distributed)
NA = not applicable (insufficient data)

Reference ranges are calculated from percentiles of the observed distributions. Calculated in February 2013 by CLEH Statistics, for studies started between March 1998 and June 2012.

Data from this study are included in the historical control data. As the concurrent vehicle controls fall close to the median and mean values for the historical control data, inclusion of the concurrent study is considered to have no effect on data interpretation.

Historical ranges for duodenum consist of only two data sets, one of which was generated in this study, and have therefore not been presented in this report.

Appendix 2
Calculation of mutation frequency and titre

Total plaques

The total number of plaques is estimated by scoring the titre plates manually. Each plaque is marked on the base and recorded on a tally counter if necessary. The plates may be checked by a second person to ensure no plaques were unmarked.

Titre

Titration involved diluting a 10 µL aliquot to 200 µL and plating 10 µL of this onto each of three petri dishes. The total number of plaques (T) is the sum total of all three plate counts. This total number of plaques (T) were present in 1.5 µL of the original reaction and 1.5 µL represents 0.15% of 1000 µL (total reaction volume).

Total titre, or number of pfu in original reaction:

$$\frac{100}{0.15} x\ T = 667 x\ T$$

Mutation frequency

The total volume available for selection plating is 10 µL less than the original reaction mix, which is 990 µL. The total number of pfu available must be corrected:

$$667\ x\ T\ x \frac{990}{1000} = 660\ x\ T$$

Finally MF is calculated by dividing the number of mutants on the three selection plates (M) by the total number of pfu available for selection.

$$MF = \frac{M}{660\ x\ T}$$

The final figure is usually multiplied by 10^6 and presented as mutants per 10^6 pfu.

Appendix 3
Certificate of analysis

International Flavors & Fragrances Inc

IFF

CERTIFICATE OF ANALYSIS

Date: 05/16/2012

Sample ID (IPC): 00198880 Lot#: 0004489289

Expiration Date: 02/2013

Customer: Covance Laboratories

Test	Test Data
Appearance	LIQUID
Color	Dark yellow
Flavor/Odor	CONFORMS TO STANDARD
Specific Gravity @ 20 C	1.0950 - 1.1050
Refractive Index @ 20 C	1.5410 - 1.5480
Purity (by GC)	Greater than 99%

Uma Parasar

Uma Parasar
Senior Research Investigator,
R&D Flavors

Appendix 4
Minor deviations from protocol

Protocol section	Subject	Deviation
Test system	Animal age	Some animals were 11 weeks old and therefore marginally outside of the 8-10 week age range stated in the protocol. All animals were considered to be young adults and of an appropriate age for mutation assessment. This deviation had no adverse impact on study integrity.
Test system	DNA extraction and DNA packaging kits	The protocol specified that DNA extraction and DNA packaging kits would be purchased from Stratagene Ltd, Cambridge, UK. However, as Stratagene have been bought by Agilent Technologies UK Ltd, Stockport, UK, the updated supplier information has been reported. This deviation has no adverse effect on study integrity.
Methods	Body weights	In addition to the body weights described in the protocol, body weights of the Main Experiment animals were also recorded on Day 29. This additional data measurement did not affect the integrity of the study.

FCF 10020039

Hasl
04/09/13
US POSTAGE $003.12

ZIP 20006
011D11622628

FEMA 100
A Century of Great Taste

Flavor and Extract Manufacturers Association of the United States
1620 I Street, NW, Suite 925
Washington, DC 20006